

Non-founder mutations in the *MEFV* gene establish this gene as the cause of familial Mediterranean fever (FMF)

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Familial Mediterranean fever (FMF) is an autosomal recessive disorder characterized by recurring attacks of fever and serositis. It affects primarily North African Jews, Armenians, Turks and Arabs, in which a founder effect has been demonstrated. The marenosttrin-pyren-encoding gene has been proposed as a candidate gene for the disease (*MEFV*), on the basis of the identification of putative mutations clustered in exon 10 (M680V, M694I, M694V and V726A), each segregating with one ancestral haplotype. In a search for additional *MEFV* mutations in 120 apparently non-founder FMF chromosomes, we observed eight novel mutations in exon 2 (E148Q, E167D and T267I), exon 5 (F479L) and exon 10 (I692del K695R, A744S and R761H). Except for E148Q and K695R, all mutations were found in a single chromosome. Mutation E148Q was found in all ethnic groups studied and in association with a novel ancestral haplotype in non-Ashkenazi Jews (S2). Altogether, these new findings definitively establish the marenosttrin-pyren-encoding gene as the *MEFV* locus.

INTRODUCTION

Familial Mediterranean fever (FMF, McKusick 249100) is an inherited disorder characterized by attacks of fever and serositis (1). It is transmitted in an autosomal recessive pattern, and affects mostly ethnic groups living around the Mediterranean basin: non-Ashkenazi Jews, Armenians, Turks and Arabs. The gene

responsible for FMF (*MEFV*) has been mapped to the short arm of chromosome 16 (2), and the gene interval was narrowed progressively to a very small interval of 60 kb at the time the gene was identified (3). Haplotype analysis led to the identification of several founder haplotypes: haplotype S in North African Jews; haplotypes ARM1, ARM2 and ARM3 in Armenians, haplotype T in Turks, haplotypes ARA1 and ARA2 in Arabs from the Maghreb, and haplotype D in Druzes. The S, ARM1, T and ARA1 haplotypes share a common origin, that we termed MED, and haplotypes D and ARM3 were also shown to be related (3,4). (These haplotypes were also described by the International FMF consortium, which designated them using a different nomenclature, shown in Figure 1.)

Gene and mutation screens in the critical interval pointed to the marenosttrin-pyren-encoding gene as a candidate for FMF (5,6). The name marenosttrin was chosen from the Latin name of the Mediterranean sea, and pyren refers to the fever. Several arguments supported the hypothesis that this gene is responsible for FMF. First, four missense mutations were identified in homozygous representatives of each ancestral haplotype. Second, there is a perfect correlation between the affected phenotypes observed across the generations and the DNA alterations in all the kindreds we analysed. Third, we found none of these sequence modifications in either non-carrier or other control individuals. Fourth, none of the other sequence variations that we observed in other genes contained within the FMF interval were disease associated.

The sequence of marenosttrin-pyren exhibits significant similarities with the *cip*-domain proteins, the prototype of which was first described in the RET finger protein (7). This domain has also been observed in other proteins for which a role in regulating gene expression has been suggested (8), or for which involvement in the

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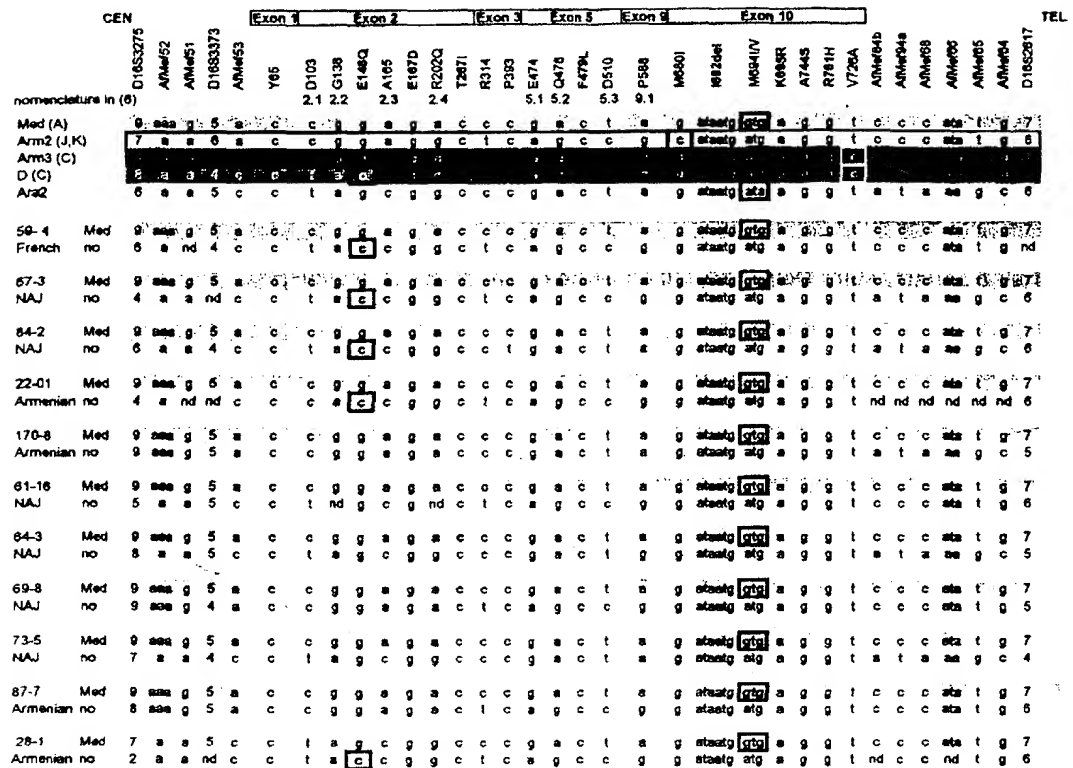


Figure 1. Sequence alterations detected by sequencing in 22 FMF individuals. Nucleic acids are in lower case and amino acids in upper case. The first line depicts the *MEFV* gene exon positions. The second line depicts the sequence variations within the gene (centre), or in flanking genetic markers (left, centromeric markers; right, telomeric markers); the novel mutated sites are in bold. The third line depicts the nomenclature used in ref. 6 for the intragenic polymorphisms. The five following lines correspond to the founder haplotypes described in ref. 5, with the alternative nomenclature used in ref. 6 in parentheses. Each founder haplotype is highlighted with distinctive shading. The lines below show the sequence variations observed in 22 individuals. In individual 29-3, the complete coding sequence of the non-founder carrier chromosome was obtained from the father. Non-Ashkenazi Jews are denoted NAJ. When the phase is undetermined, the corresponding markers are shown with a slash (for example c/t for individual 21-31 at position R314). 'no' denotes chromosomes that are distinct from the previously described founder haplotypes. Mutations are boxed.

regulation of immune-related protein expression has been proposed (9,10). Transcription of the *MEFV* gene has only been reported in granulocytes (6), which play an essential role in the immune response. Thus, these data also support a role for this gene in the recurring attacks of inflammation observed in FMF patients.

Because all these founder mutations were rather conservative and occurred in the founder haplotypes only, one could not exclude that these were amino acid variations in complete linkage disequilibrium, with no phenotypic effect. We therefore undertook a search for novel mutations in genomic DNA, using primers flanking each exon of the *MEFV* gene and sequencing completely the amplicons in 22 FMF-affected patients. We also performed an extensive study of exon 10 in 120 non-founder carrier FMF chromosomes. Eight novel mutations which are associated with the FMF disease were identified. Six of these novel mutations were not found in control chromosomes. Our results thus establish that *MEFV* is the causative

gene for FMF. In addition to disease-causing mutations, several polymorphisms were also detected. These polymorphisms were used as genetic markers to refine the haplotype analysis of FMF-related haplotypes. One new founder haplotype associated with the E148Q mutation was found.

RESULTS

Sequence alterations

To identify novel mutations, we first focused on 22 individuals in whom at least one carrier chromosome did not bear any of the four common founder mutations previously described in exon 10 (5,6). The genomic organization data were used to design five primer sets covering the 10 *MEFV* exons and their boundaries. Genomic DNA samples from FMF patients were amplified, and

144-1	Med	4	a	a	5	c	c	t	a	g	c	g	g	c	c	c	a	g	c	c	g	g	atstg	atg	a	g	g	t	c	c	ata	t	g	7			
NAJ	no	8	a	a	5	c	c	t	a	g	c	g	g	c	c	c	a	g	c	c	g	g	atstg	atg	a	g	g	t	c	c	ata	t	g	7			
51-1	Med	2	a	nd	5	a	c	c	g	g	a	g	g	g	c	t	c	a	g	c	g	g	atstg	atg	a	g	g	t	t	a	a	g	c	nd			
French	no	7	a	nd	5	c	c	t	a	g	c	g	g	g	c	t	c	a	g	c	g	g	atstg	atg	a	g	g	t	t	a	a	g	c	nd			
149-3	Med	4	a	a	5	c	c	t	a	g	c	g	g	g	c	c	c	g	a	c	t	a	g	atstg	atg	a	g	g	t	a	a	g	g	4			
NAJ	no	7	a	a	4	c	c	t	a	g	c	g	g	g	c	c	c	g	a	c	t	a	g	atstg	atg	a	g	g	t	a	a	g	g	4			
123-1	D	9	a	a	4	c	c	t	a	g	c	g	g	g	c	c	c	g	a	c	t	a	g	atstg	atg	a	g	g	t	a	t	a	a	g	c	8	
Druze	no	5	a	a	4	c	c	t	a	g	c	g	g	g	c	c	c	g	a	c	t	a	g	atstg	atg	a	g	g	t	a	t	a	a	g	c	8	
21-11	Arm3	7	a	a	5	a	c	c	g	g	a	g	g	g	c	c	c	g	a	c	t	a	g	atstg	atg	a	g	g	t	nd	t	a	a	g	c	5	
NAJ	no	7	a	a	5	a	c	c	g	g	a	g	g	g	c	c	c	g	a	c	t	a	g	atstg	atg	a	g	g	t	nd	t	a	a	g	c	5	
92-3	Arm3	7	a	a	6	a	c	c	g	g	a	g	g	g	c	t	c	a	g	c	c	g	g	atstg	atg	a	g	g	t	c	c	ata	t	g	8		
Armenian	no	7	a	a	6	a	c	c	g	g	a	g	g	g	c	t	c	a	g	c	c	g	g	atstg	atg	a	g	g	t	c	c	ata	t	g	8		
29-3	Arm3	6	a	a	nd	5	a	c	c	g	g	a	g	g	c	at	c	g	a	g	Uc	g	g	atstg	atg	a	g	g	t	a	c	c	nd	g	nd	5	
Armenian	no	6	a	a	nd	5	a	c	c	g	g	a	g	g	c	at	c	g	a	g	Uc	g	g	atstg	atg	a	g	g	t	a	c	c	nd	g	nd	5	
21-31	NAJ	no	8	a	a	6	a	c	c	g	g	a	g	g	c	at	c	g/a	a/g	c	Uc	a/g	g	atstg	atg	a	g	g	t	a	t	a	a	g	nd	N	
NAJ	no	8	a	a	6	a	c	c	g	g	a	g	g	g	c	at	c	g/a	a/g	c	Uc	a/g	g	atstg	atg	a	g	g	t	a	c	c	ata	t	g	N	
98-1	no	10	a	a	g	5	a	c	c	g	g	a	g	a	c	t	c	a	g	c	c	g	g	atstg	atg	a	g	g	t	c	c	c	ata	t	g	6	
Turkish	no	8	a	a	5	c	c	t	a	g	c	g	g	g	c	t	c	a	g	c	c	g	g	atstg	atg	a	g	g	t	c	c	c	ata	t	g	6	
86-4	no	9	a	a	nd	5	a	c	c	g	g	a	g	g	c	t	c	a	g	c	c	g	g	atstg	atg	a	g	g	t	c	c	c	ata	t	g	nd	
French	no	9	a	a	nd	5	a	c	t	a	g	c	g	g	a	c	t	c	a	g	c	c	g	g	atstg	atg	a	g	g	t	c	c	c	ata	t	g	nd
49-1	no	1	a	nd	4	c	c	t	a	g	c	g	g	g	c	at	c	g/a	a/g	c	Uc	a/g	g	atstg	atg	a	g	g	t	a	t	a	a	g	c	nd	
French	no	6	a	nd	5	c	c	t	a	g	c	g	g	g	c	at	c	g/a	a/g	c	Uc	a/g	g	atstg	atg	a	g	g	t	a	t	a	a	g	c	nd	

Figure 1. Continued

screened for the presence of DNA alterations by sequencing. Eighteen nucleotide variations were identified by this approach, as shown in Figure 1. Ten polymorphisms corresponded to silent substitutions: Y65 TAC/TAT (exon 1), D103 GAT/GAC, G138 GGA/GGG and A165 GCA/GCC (exon 2), R314 CGC/CGT and P393 CCC/CCT (exon 3), E474 GAG/GAA, Q476 CAA/CAG and D510 GAT/GAC (exon 5), and P588 CCA/CCG (exon 9) [numbering of residues according to (6)]. These polymorphisms were used as biallelic markers to consolidate the haplotype analysis (see below). Among the other alterations, three corresponded to one of the mutations previously described (5), and five corresponded to novel missense substitutions, namely E148Q (GAG/CAG), E167D (GAG/GAC), R202Q (CGG/CAG), T267I (ACA/ATA) and F479L (TTC/TTG).

To test other patients on a larger scale, we then developed a denaturing gel gradient electrophoresis (DGGE) screening test focused on the tenth exon, which is the preferential target of mutations in FMF patients. This test allowed rapid and accurate distinction of the four previously described mutations, except for M680I and V726A homozygotes (Fig. 2a). A total of 120 chromosomes from definitive or probable FMF patients with less than two of the known exon 10 mutations were screened with this test, and in some rare cases a distinct pattern was observed (Fig. 2b). The corresponding samples were sequenced, and we identified two novel silent substitutions (P706 CCG/CCA and S683 TCG/TCA), three novel missense substitutions (K695R AGG/AAG, A744S GCC/TCC and R761H CGT/CAT) and one deletion (I692del: the codons ATA.ATG corresponding to I692 and M693 were transformed into a single ATG codon after deletion of the AAT triplet). Mutation K695R was found in three Jewish families (16, 108 and 201), whereas the other mutations were found only once. A schematic diagram summarizing the

novel mutations reported in this report, as well as the mutations previously reported, is shown in Figure 3.

We also extended the search for the novel exon 2 mutations to this larger set of patients. This was performed essentially by restriction fragment length polymorphism (RFLP) analysis (the restriction sites used to confirm mutations are listed in Table 1). We found no other E167D or T267I substitutions. In contrast, the E148Q mutation was found in 29/120 (24%) of FMF chromosomes with no exon 10 mutation. This mutation was seen in patients with several distinct origins: Afghan, Arab, Armenian, Druze, French, non-Ashkenazi Jewish and Turkish (Fig. 4).

Most patients with the novel mutations reported here were compound heterozygotes (Table 2), with the second mutation being one of those described previously (5,6). Three haplotypes combining two allelic mutations were observed: all D chromosomes presented an E148Q mutation in addition to V726A, the patient harbouring the I692del deletion also had an E148Q substitution on the same chromosome, and one chromosome of patient 29-3 carried E167D in addition to F479L.

Controls

To evaluate whether these substitutions corresponded to polymorphisms or were novel mutations, we screened a set of control DNA samples for each of them. The R202Q (CGG/CAG) substitution was found frequently among non-carrier chromosomes as well as in controls unrelated to FMF families: it was found in 20% of chromosomes in unrelated individuals from the CEPH panel, in 9/56 (15%) of non-carrier chromosomes studied in the FMF families and 19/123 (16%) of carrier chromosomes without exon 10 mutation ($P = 0.9$, not significant). Therefore, this substitution probably represents a common polymorphism of the marenostriin/pyrin-encoding gene.

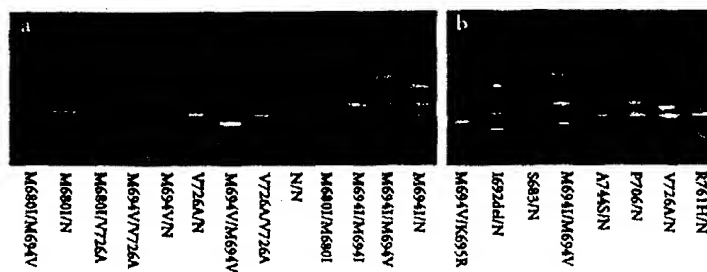


Figure 2. DGGE analysis of *MEFV* exon 10. (a) Migration patterns obtained with genotypes previously described in ref. 5. (b) Migration patterns obtained with new genotypes.

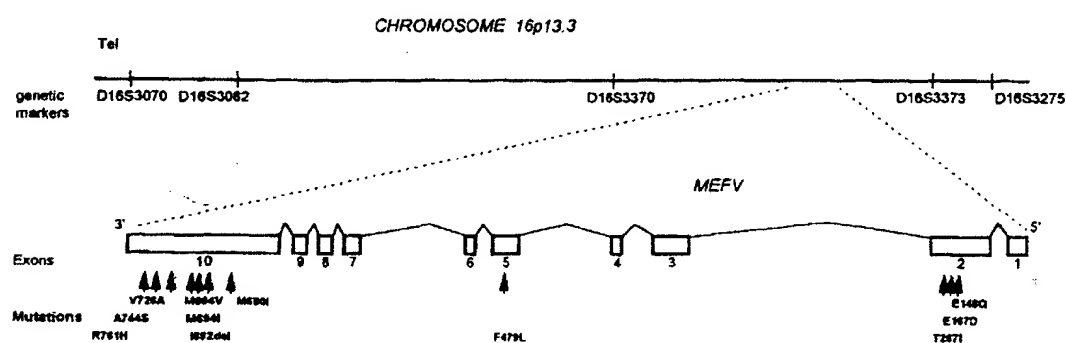


Figure 3. Schematic diagram of the *MEFV* gene depicting the location of the various mutations found. Exons are numbered 1–10, and are drawn to scale.

Table 1. Description of the marenostriin/pyrin mutations and corresponding frequency in control populations

Family/families	Codon change	Amino acid	Position	Exon	Restriction site	Mutation frequency in control chromosomes CEPH	Non-carrier
See Figure 4	GAG→ACG	Glu→Gln	148	2	AraI loss	1/148	2/131
29	GAG→GAC	Glu→Asp	167	2	EcoD109I loss	0/148	0/57
21	ACA→ATA	Thr→Ile	267	2	MspAII loss	0/148	0/57
29	TTC→TTG	Phe→Leu	479	5	7th111111 creation (enzyme not available)	0/130	0/51
ARM2 haplotype- related families	ATG→ATC	Met→Ile	680	10	HinfI loss	0/146	>250
240	AAT deletion (from codons ATA-ATG)	Be del	692	10	none	0/146	0/52
MED haplotype- related families	ATG→GTG	Met→Val	694	10	none	0/146	>250
ARA2 haplotype- related families	ATG→ATA	Met→Ile	694	10	none	0/146	>250
16, 108, 201	AAG→AGG	Lys→Arg	695	10	MnI creation	0/146	1/52
D and ARM3 haplotypes- related families	GTT→GCT	Val→Ala	726	10	Ahd creation	0/146	>250
113	GCC→TCC	Ala→Ser	744	10	none	0/146	0/52
231	CCT→CAT	Arg→His	761	10	Eco722I loss	0/146	0/52

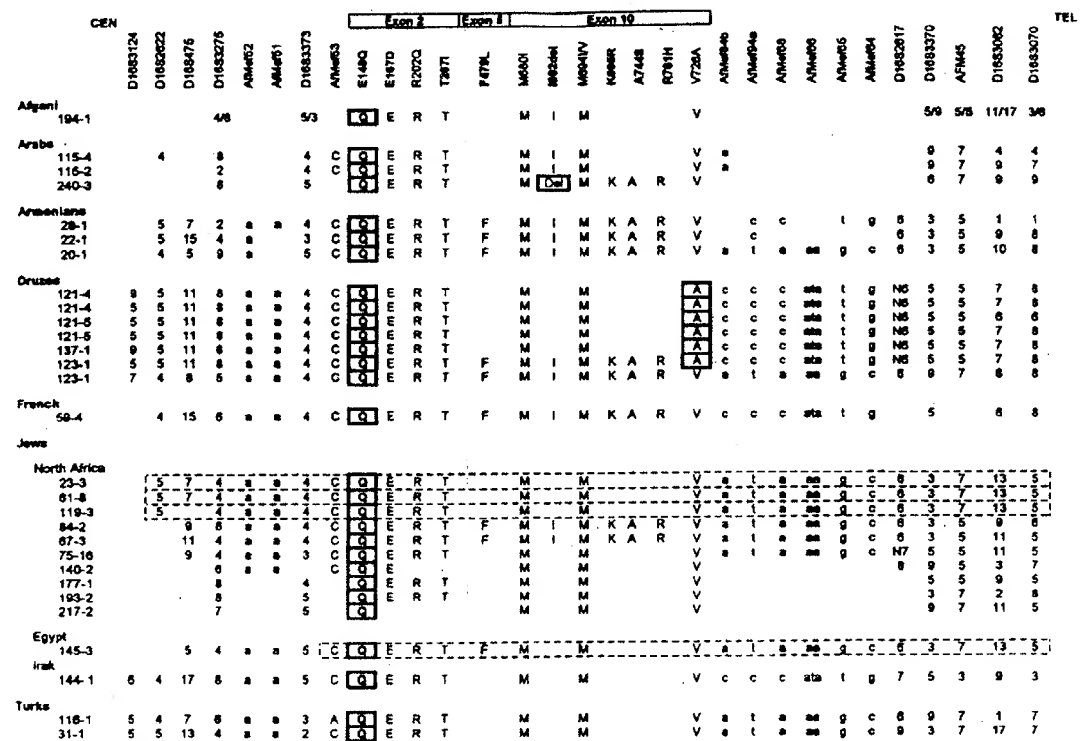


Figure 4. Haplotypes associated with the E148Q mutation. The new founder haplotype is termed haplotype S2. Nucleic acids and amino acids are depicted as in Figure 1.

The E167D, T267I, F479L, I692del, A744S and R761H sequence variants were absent from a panel of DNA from 120–148 chromosomes of the CEPH families and a large number of validated non-carriers from FMF families (Table 1). They probably represent mutations of the marenostin/pyrin-encoding gene. The E148Q substitution was found once in a CEPH individual, but this occurrence could be interpreted as the presence of a mutated chromosome in a heterozygous healthy carrier. It was also found in three non-symptomatic FMF patients' parents, once associated with V726A both *in cis* and *in trans*, once with a K695R mutation on the other chromosome, and once with an as yet unknown mutation. Since the distribution of the E148Q substitution was significantly higher (Fisher's exact test, $P < 10^{-4}$) in chromosomes from FMF patients (29/120) than in chromosomes from symptom-free individuals (3/131), this absence of symptomatology could be explained by low penetrance of E148Q or of the accompanying mutation. The K695R substitution was never found in the CEPH panel, but was found twice in asymptomatic individuals from FMF families. This substitution may thus also correspond to a mutation with incomplete penetrance.

Haplotype analysis

The E148Q mutation was the most frequent substitution among the tested non-founder FMF carrier chromosomes (24%). To discriminate between a founder effect and a *de novo* recurrence, disease-associated haplotypes across the FMF region were determined by typing neighbouring markers.

Genotype analysis of families carrying the E148Q mutation showed that this mutation segregated with the same alleles from D16S2622 to D16S3275 in four patients (23-3, 61-8, 119-3 and 145-3) out of 29 examined (Fig. 4). The existence of a conserved haplotype and the fact that this haplotype was only found in Jews suggested that these patients have inherited the same disease allele from a common ancestor. We named this new haplotype S2 in reference to haplotype S, the most frequent founder haplotype in Jews (5). Moreover, allele 13 of the D16S3082 marker has never been observed in any other carrier ($n > 400$) or non-carrier chromosomes ($n > 250$) in the studied populations, whereas it was systematically associated with E148Q (4/29 versus 0/400, Fisher's exact test $P < 10^{-4}$).

Table 2. Genotypes of individuals* with at least one of the eight new MEFV mutations

	Exon 2			Exon 5	Exon 10					Unknown
	E148Q	E167D	T267I	F479L	I692del	M694V	K695R	V726A	A744S	R761K
DR: 121-4 121-5 121-9*	++							++		
DR: 137-1	+							++		
DR: 123-1	++							+		
ARM: 29-3		+		+				+		
ARA: 240-3	+				+					
ARA: 115-2	++									+
ARM: 20-2 22-1 28-1	+									
TK: 31-1 116-1 F: 59-4										
NAJ: 16-3 23-3 61-8 67-3						+				
75-16 84-2 119-3 140-2										
144-1 177-1 217-2										
NAJ: 16-2*	+						+			
ARA: 99-1* 115-4	+									
NAJ: 145-3 193-2 AF: 194-1	+									+
NAJ: 21-31			+							+
ARM: 231-1							+		+	
NAJ: 16-4 108-4 201-2*						+	+			
ARA: 113-1									+	+

*Patients are denoted by their family-individual number. Their origin is schematized as follows: AF, Afghan; ARA, Arabs; ARM, Armenians; DR, Druze; F, French; NAJ, non-Ashkenazi-Jews; TK, Turks. Patients with definitive or probable FMF according to Tel Hashomer criteria are shown in bold or italic letters, respectively. Those with no symptoms (all were above 30) are depicted with an asterisk.

E167D and F479L in individual 29-3 and E148Q and I692del in individual 240-3 are located on the same chromosome.

We previously reported that the Druze haplotype shared a common segment with the Arm3 haplotype (3,4). This was emphasized particularly by the identification of the same mutation detected in the *MEFV* gene at position 726 (Val→Ala). Analysis showed that most of the polymorphisms were indeed conserved between the two haplotypes. However, this conservation is clustered distal to E148Q, which is absent in Arm3 chromosomes. This position therefore defines the centromeric limit of the common segment.

DISCUSSION

The marenostriin/pyrin-encoding gene has been identified as the most likely target for mutations which cause FMF (5,6). Four different germline mutations have been found in the coding region of this gene previously, and 86–93% of the FMF chromosomes reported to date have been shown to have one of these mutations. Each of these mutations has been linked to a founder haplotype. Despite numerous functional and genetic arguments, one could still argue that each of the previously reported mutations described in this gene could be simply a polymorphism in complete linkage disequilibrium with one of the founder haplotypes. We have now investigated mutations in the marenostriin/pyrin-encoding gene in FMF patients for which at least one 16p13.3 chromosome did not bear any previously described founder mutations. In typical cases that we had

analysed to identify the *MEFV* gene, the more frequent mutation (M694V) was shown to be a severe one, at least in a homozygous state (16,17). To increase the chances of detecting novel mutations, we enlarged the present study to atypical cases i.e. patients suffering from 'probable FMF', according to Tel Hashomer criteria (18) or patients from groups rarely affected by FMF (e.g. French). This led to the identification of several novel sequence alterations which were not observed in control chromosomes. Our observation of these *MEFV* mutations in FMF patients validates the initial reports of the French (5) and of the International Consortia (6) and further establishes this gene as the cause of FMF.

We report a total of eight novel mutations in our FMF samples. Seven of them are missense mutations, and we detected no drastic mutations, such as nonsense, frameshift or splice mutations. The nature of the substitutions is also compelling: all mutations so far described in the tenth exon are conservative (M680I, M694V, M694I, K695R, V726A, A744S and R761H), and the deletion I692del conserves the reading frame. These mutations are all located in the rfp C-terminal domain (7). Conversely, less conservative alterations (E148Q, E167D, T267I and F479L) are tolerated in the most N-terminal part of the protein, which also bears a non-conservative amino acid polymorphism (R202Q). This suggests that more drastic mutations—particularly those affecting the C-terminal region—could be lethal or would induce

a very different phenotype in homozygous individuals. Hence the chance of observing such mutations might be very low in typical FMF patients. Similar genetic heterogeneity has been described for other genes, such as the receptor tyrosine kinase gene *RET* (19). It thus could be hypothesized that functional constraints imposed on the C-terminal region also restrict the nature of the mutations occurring in the rest of the protein. So far, only the M694 residue has been described as the target of multiple mutations (M694V, M694I and, very recently, in an English patient, M694del) (5,20).

Apart from the previous mutations described in exon 10, the E148Q mutation was the most frequently observed in our samples. In non-Ashkenasi Jewish patients, this substitution even appears to be the second most frequent genetic alteration responsible for FMF after M694V. The study of markers flanking or internal to the *MEFV* gene led to the identification of a specific haplotype associated with this mutation in this population. However, it should be stressed that this mutation was also observed in several other chromosomes not harbouring any conserved haplotype, and in all populations studied. This suggests that this position is a preferential site for recurrent mutations.

Three non-symptomatic individuals were identified for which both alleles of the marenostrin/pyrin-encoding gene were affected by mutations. It has been proposed previously that the penetrance of FMF is incomplete, especially in females, since the female/male ratio of affected individuals is <1 (17). The V726A substitution has been suggested to be a milder mutation than M694V (6). The detection of symptom-free patients with some of the newly reported mutations was thus not unexpected.

Among the carrier chromosomes described here, several presented double mutations in the marenostrin/pyrin-encoding gene. An E148Q mutation was present in all D haplotypes in addition to a V726A mutation, and in one chromosome with an I692del; one chromosome of patient 29-3 presented both E167D and F479L mutations. Double mutations in the same gene have been described in other diseases, such as sickle cell anaemia (22), and it has been proposed that this occurred by homologous recombination between an already mutated allele and an allele presenting a frequent mutation (the E6V HbS allele in sickle cell anaemia, for example). A similar explanation could be proposed for the E148Q/V726A double mutation, since these mutations are also very frequent in the populations we studied. It has also been reported that double substitutions could be linked to distinct disease phenotypes (23) but, in our case, more patients with such genotypes are needed to draw any conclusions.

Sequence analysis of the coding region of *MEFV*, including splice site junctions, failed to detect anomalies in 17 chromosomes out of 45. This low proportion of mutations identified could be explained in several ways. First, the mutations could lie either in the promoter region, within an intron, or in the 3'-untranslated region. Second, a major deletion or translocation, which would not be detectable by the genomic PCR/sequencing approach (24), could cause inactivation of the gene. Other mutation detection techniques, such as Southern blot, genomic long-range PCR, RT-PCR analysis or a fluorescence *in situ* hybridization (FISH) approach are needed to identify such alterations in the remaining families. Third, it may be that some of the cases studied, for which no mutations were detected (three cases), are not linked to the 16pter locus, as genetic heterogeneity has been reported for FMF, at least in the Turkish population (25) (in our case, the small size of these particular families hampered

linkage analysis). Lastly, we cannot exclude the possibility that exon-located mutations were missed by the sequencing method we used, as one of the allele could be amplified or sequenced preferentially, producing no apparent double peak.

The clustering of the founder mutations in exon 10 of the marenostrin/pyrin-encoding gene led us to hope that the search for additional mutations and the identification of at-risk individual would be simplified (5). However, as shown in this work, mutations can in fact be observed in other exons, especially in patients with milder forms of FMF or of non-Mediterranean ancestry. However, the spectrum of mutations leading to FMF may provide some insight into the role of the marenostrin/pyrin protein, and additional information about the mechanism involved in the symptoms.

MATERIALS AND METHODS

Pedigrees and DNA isolation

This study included 247 individuals from FMF families (of Afghan, Arab, Armenian, Druze, French, Jewish or Turkish origin) and 74 unrelated parents or grandparents from CEPH families from various Caucasoid groups (11). This study was approved by the appropriate institutional review board (Comité Consultatif pour la Protection des Personnes se Prêtant à des Recherches Biomédicales), and informed consent was obtained from each subject. DNA was isolated from circulating lymphocytes, or from Epstein-Barr virus-transformed lymphocytes, using standard procedures (12).

Haplotype analysis

Patients presenting novel mutations, together with the available parents and unaffected siblings, were genotyped with at least six microsatellite markers (*D16S3070*, *D16S3082*, *AFM45*, *D16S3370*, *D16S3373* and *D16S3275*) and some families were completed with single nucleotide polymorphism markers (SNP: *AFMef64*, *AFMef65*, *AFMef66*, *AFMef68*, *AFMef94a*, *AFMef84b*, *AFMef53*, *AFMef51* and *AFMef52*) that surround the FMF locus. The oligonucleotide primers, the PCR amplification conditions and the allele numbering process have been described elsewhere (3,13). Haplotypes were determined for affected individuals after the derivation of phase from the parental genotypes.

Exon amplification

The 10 exons of the *MEFV* gene and their flanking intronic sequences were amplified from genomic DNA. The PCR used the following primers: 5'-GCCTGCTTACCTATCTCCCTTCCCC-TCCC-3' and 5'-CACCCCTTCCCCCGTCCCTGTGCC-3' (exon 1 and 2), 5'-CACAGCAGAATCTCGGGG-3' and 5'-GCA-CATCTCAGGCAAGGG-3' (exon 3 and 4), 5'-GCCCTTC-TCCCTATCAAATCC-3' and 5'-CCACCTCTTATCCACCT-CC-3' (exons 5 and 6), 5'-CGGAGAGCCCCAGAACATGATACAGC-3' and 5'-GCCAAAGTTGAGGACCAGCATTGAGACC-3' (exons 7, 8 and 9), 5'-GGAGCACCTGAGAGTGCCACCCACC-3' and 5'-ATTGGCGCTCAGGCACAT-3' (exon 10). The 50 µl reactions were carried out under standard conditions, containing 200 ng of genomic DNA, 50 mM KCl, 1.5 mM MgCl₂, 1 mM each of forward and reverse primers, 2.5 U of *Taq* polymerase, 250 mmol of dNTP, 0.1% Triton and 10 mM Tris (pH 8.8) as follows: an initial denaturation at 95°C for 5 min

followed by 35 cycles of denaturation at 95°C (15 s), annealing at 53–65°C (30 s, temperature depending on the primers used), and extension at 72°C (3 min), with a final 10 min extension at 72°C.

Sequencing

PCR products were purified using Sephadex P100 gel filtration, sequencing reactions were carried out using the ABI PRISM dye terminator cycle sequencing kit, and the products were analysed on an ABI 377 DNA sequencer. DNA sequencing was performed in both directions, initiated from the forward and reverse primers used in the initial PCR of each exon, and with the following additional primers: 5'-TCCCCAGGTCAGAGTGAG-3' and 5'-CTTGCCCATCTGTCTGT-3' (exon 1); 5'-GGAAGCCCCCTGAGCAAAC-3', 5'-GTTTATAGAGATGGCGGGG-3', 5'-GGTTCGTGTGCCGAGTC-3' and 5'-GTGGGACAGCTTCATCATTTTG-3' (exon 2); 5'-CACCAACAACCCAGAGT-3' and 5'-CAGGAATCACGCACACAGG-3' (exon 3); 5'-CTCCCTCCTCTTCCTG-3' and 5'-ACGATGAGCCCATCTGCC-3' (exon 4); 5'-CTTCACCCACTTGTTC-3' (exon 5); 5'-CACTTCCACTGACACCCT-3' (exon 6); 5'-CAGCATTTAGACCTCTGAATCC-3' and 5'-GATGGAGGAGAGGTTGA-3' (exon 7); 5'-TCAACCTCTCCTCCATC-3' and 5'-TCAAGTCAACAGCACAAG-3' (exon 8); 5'-GAGGAACGGGATTATAC-3' and 5'-CGGAGAGCCCAACAATGATACAGC-3' (exon 9); and 5'-CTGCTATAATCGGGTAGGCTC-3', 5'-TATCATTGTCTGGGCTC-3', 5'-CTACCTGTCCCTGTTTCC-3' and 5'-AAAGGAGATGCTTCCAAC-3' (exon 10). Nucleotide changes in heterozygous individuals were detected with Polyphred (14), and checked by manual inspection of characteristic double peaks.

In several kindreds where sequence variation was found in one sample selected for investigations, members of the family for whom DNA was available were analysed for the putative mutation identified in the initial samples, in order to study the segregation of the mutation with FMF.

Denaturing gel gradient electrophoresis (DGGE)

Exon 10 was also screened for mutations using DGGE. The clamped primer 5'-CGCCCCCGCGCCCCCGCCCCGCGCCGCGCCCCCGCGAGAGCAGGAAGAGAGATGC-3' was used together with 5'-TATCATTGTTCTGGGCTC-3' for PCR amplification. Denaturing conditions resulting in optimal resolution were selected by use of Lerman's MELT87 and SQHTX programs (15). The amplification products were subjected to electrophoresis on a 6.4% polyacrylamide gel containing a linearly increasing denaturant concentration of 30–80%. The gels were run at 160 V and 60°C for 6 h.

Restriction enzyme analysis

Mutations in exon 2 were analysed by restriction enzyme tests, after amplification with the 5'-GGTTCGTGTGCCGAGTC-3' and 5'-GTGGGACAGCTTCATCATTTTG-3' primers which produced a 595 bp fragment. The G→C transversion at codon 148 suppressed an *Ava*I restriction endonuclease recognition site, resulting in the replacement of two fragments of 69 and 221 bp by a 290 bp fragment in the mutated chromosomes. The G→C transversion at codon 167 suppressed an *Eco*01091 site, resulting

in the replacement of two fragments of 179 and 268 bp by a single 447 bp fragment. The G→A transition at codon 202 created a *Pvu*II site, which replaced a 566 bp fragment with two fragments of 196 and 370 bp. The C→T transition at codon 267 suppressed an *Msp*AI site, and replaced two fragments of 29 and 174 bp with a single 203 bp fragment. All digestions were performed according to the manufacturer's recommendations (New England Biolabs, Eurogentec), and restriction products were analysed on a Nusieve 3% Seakem/1% agarose gel or on a non-denaturing 5% or 8% polyacrylamide gel.

Exon 5, harbouring the C→G transversion at codon 479, was amplified with the 5'-CTTCACCCACTTGTTC-3' and 5'-CCACCTCTATCCACCTCC-3' primers which produced a 435 bp fragment. Since no restriction enzyme was available, this substitution was analysed by sequencing using the same primers.

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